7.9.2015

MONDAY, 9/7

Petra

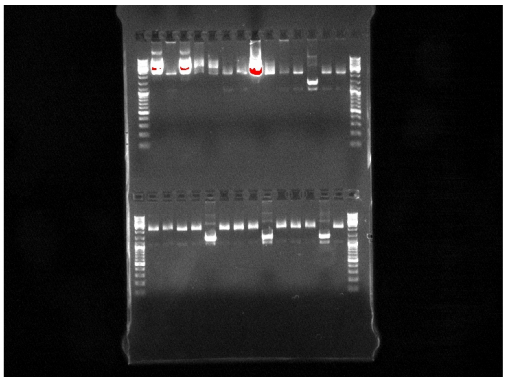
Making o/n cultures:
Made o/n cultures of AtoB and CenA Gibson colonies 6-10 (the same ones that were unsuccesfully screened with colony PCR last week).

- 2 ul cultures
- 2 µl chloramphenicol stock (stock concentration 34 mg/ml)
- 10 cultures in total (5 AtoB and 5 CenA)
- incubated in 37 C with shaking overnight

Restricted Amph (no linker) 1-19 and GFP+Amph+linker with FastDigest PstI (also used FAstDigest Green Buffer) following mostly the restriction protocol. However, used 1 ul FD PstI, the incubation was 5 min and didn't heat inactivate (as the ThermoFisher FD PstI protocol says it isn't needed).

Made a 1,3 % agarose gel with EtBr. Restricted and undigested Amph and GFP+Amph were run in the gel for 35 min with 100 V.
Pipeting order was:
I 1. ladder 2 ul 2.Undigest GFP+Amph 1 6 ul 3. Amph 1 6 ul 4. Undigest GFP+Amph 2 6 ul 5. GFP+Amph 2 6 ul

Geldoc_2015-09-07_12hr_29min.jpg



Results: It doesn't seem there are any correct sized inserts.

8.9.2015

TUESDAY, 9/8

Petra

NanoDrop results of Car Amph (2nd gibson), which were miniprepped on 4.9.:

Table3

Sample	DNA (ng/ul)	A260/A280
Car Amph 1	69,5	1,74
Car Amph 2	80,4	1,84
Car Amph 3	72,8	1,87
Car Amph 4	85,3	1,87
Car Amph 5	83,2	1,88
Car Amph 6	80,2	1,87
Car Amph 7	79,2	1,88
Car Amph 8	80,7	1,88
Car Amph 9	64,3	1,87

Miniprepping yesterday's o/n cultures:

Miniprepped AtoB Gibson 6-10 and CenA Gibson 6-10 o/n cultures using Macherey-Nagel NucleoSpin Plasmid EasyPure kit. Followed the protocol. Nanodrop results are in Table 1.

Table1

Construct	Concentration (ng/µl)	A260/A280
AtoB Gibson 6	36,7	1,89
AtoB Gibson 7	35,9	1,85
AtoB Gibson 8	46,4	1,90
AtoB Gibson 9	43,9	1,85
AtoB Gibson 10	40,2	1,91
CenA Gibson 6	46,9	1,86
CenA Gibson 7	28,5	1,85
CenA Gibson 8	43,0	1,83
CenA Gibson 9	37,3	1,86
CenA Gibson 10	40,3	1,85

Restriction digestion for AtoB and CenA:

Restricted minipreps of AtoB Gibson 6-10 and CenA Gibson 6-10 with EcoRI and Pst1. Used 2,5 µl 10x Thermo Fisher Fast Digest Green Buffer and 0,5 µl Fast digest EcoRI & Fast digest PstI for each restricrtion reaction. The amounts of DNA and water pipetted are listed in Table 2. Total reaction volume: 25 µl. Incubated the reactions in 37 C for 15 min and inactivated in 80 C for 5 min.

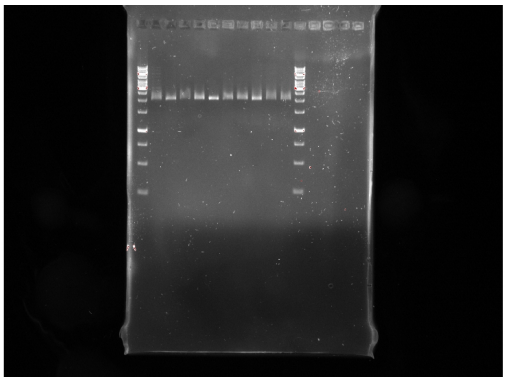
Table2

Construct	Amount pipetted for 250 ng (µl)	Water added (µl)
AtoB Gibson 6	6,8	14,6
AtoB Gibson 7	7,0	14,4
AtoB Gibson 8	5,4	16,0
AtoB Gibson 9	5,8	15,6
AtoB Gibson 10	6,2	15,2
CenA Gibson 6	5,4	16,0
CenA Gibson 7	8,8	12,6
CenA Gibson 8	5,8	15,6
CenA Gibson 9	6,8	14,6
CenA Gibson 10	6,2	15,2

Checking AtoB and CenA restrictions on gel:
Made a 1,3 % agarose gel with ETBR. Pipetted 6 µl each restriction reaction to the wells. Used Thermo Fisher O'GeneRuler 1 kb ladder. Ran the gel for 20 min, 120 V. Continued the run for additional 15 min, 100 V. Pipetting order:

1. Ladder
2. AtoB Gibson 6
3. AtoB Gibson 7
4. AtoB Gibson 8
5. AtoB Gibson 9
6. AtoB Gibson 10
7. CenA Gibson 6
8. CenA Gibson 7
9. CenA Gibson 8
10. CenA Gibson 9
11. CenA Gibson 10
12. Ladder

Geldoc_2015-09-08_17hr_53minAtob_Cena_gibson_restriction.jpg

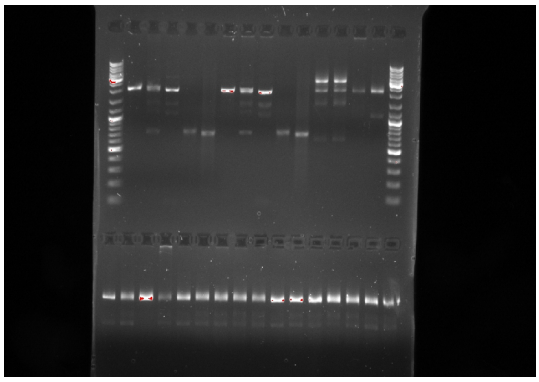


According to the gel picture AtoB Gibson 6 might contain right-sized insert (6401 bp). All the other samples seem to only contain pSB1C3 backbone (2070 bp) and no insert can be seen.

Wanted to make new amphiphilic constructs because of the gel results. Now restricted with FD enzymes mostly following the protocol: AH045 with E&S, pSB1A3 with E&P, AH045+GFP 15 with E&S, Amph+term with X&P and Amph+linker wth X&P. Also used both normal FD buffer (I) and green buffer (II) so did the same restriction twice only differing from used buffer. However, the incubation time and heat inactivation temperature and time depended on which enzyme was used. Double digestions which contained EcoRI was incubated 25 min and inactivated 10 min in 80 C and digestions which had XbaI incubated 45 min and inactivated 20 min in 65 C. Also, on 7.9. done restrictions of Amph and GFP+Amph were incubated 1 h more, because 5 min didn't seem to be enough.

Made a 1,3 % agarose gel with EtBr. The restricted construct parts and the restrictions of GFP+Amph and Amph were again run in the gel for 35 min with 100 V. Pipeting order was:
I 1. ladder 2 ul 2. pSB1A3 I 6 ul 3. AH045+GFP I 6 ul 4. AH045 I 6 ul 5. Amph+linker I 6 ul 6. Amph+term I 6 ul 7. pSB1A3 I 6 ul 8. AH045+GFP I 6 ul 9. AH045 I 6 ul 10. Amph+linker I 6 ul 11. Amph+term I 6 ul 12. GFP+Amph 1 6 ul 13. GFP+Amph 2 6 ul 14. Amph 1 6 ul 15. Amph 2 6 ul 16. ladder 2 ul
II 1. Amph 19 6 ul 2.-15. Amph 3-16 6 ul 16. Amph 18 ul

Geldoc_2015-09-08_19hr_20min_restrictions.jpg



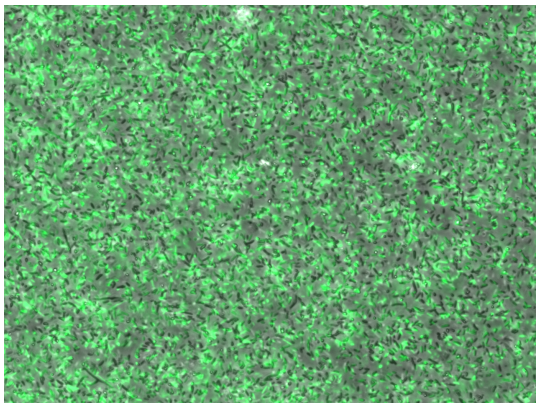
Results: The restriction seems to be done correctly.

Ligated pSB1A3 (3 ul)+AH045 (0,3 ul)+Amph+term (3,1 ul) and pSB1A3 (3 ul)+AH045+GFP (3,5 ul)+Amph+linker (3,2 ul) - constructs following the T4 ligase kit protocol (molar ratio was 3:1).
Used 5 ul of the ligations to transform them to Top10 CHEM competent cells following mostly the protocol. However, the incubation time was 20 min and incubated two nights in RT. Plated 50 ul and 150 ul from each tube (4 ligation tubes) to AMP plates.

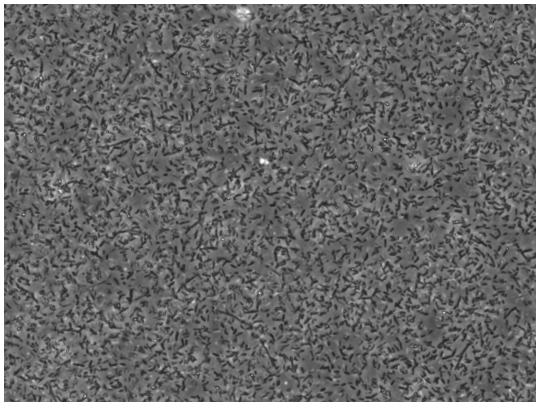
Took fluorescence microscopy pictures (x40) of *E. colis* which had GFP+Amph constructs (1 & 2; the same ones which were run in the gel). Before taking pictures, made samples for the microscope from o/n cultures of GFP+Amph. First, pelleted cells with a centrifuge for 2 min for 3000 rcf. Then resuspended the pellet with 200 ul fresh growth media, which was LB in our case. And then did 1:1000 and 1:100 dilutions with H2O.

Undiluted (directly from o/n)

Experiment-64.jpg

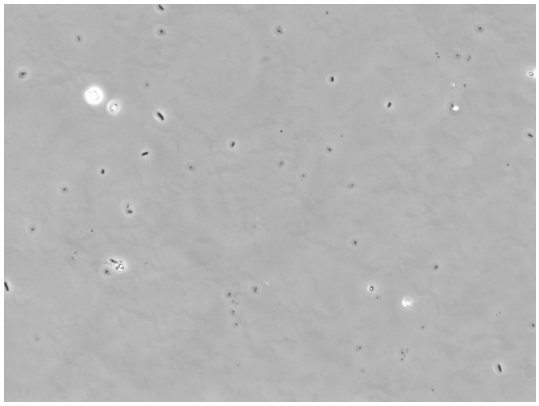


Experiment-65.jpg

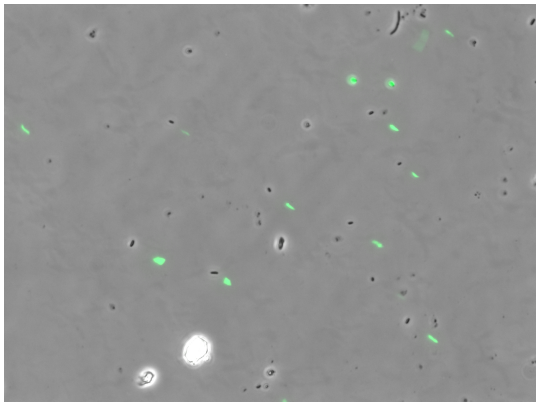


1:100

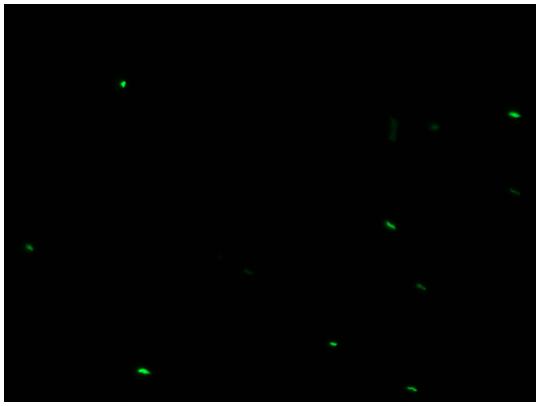
Snap-114.jpg



Snap-111.jpg

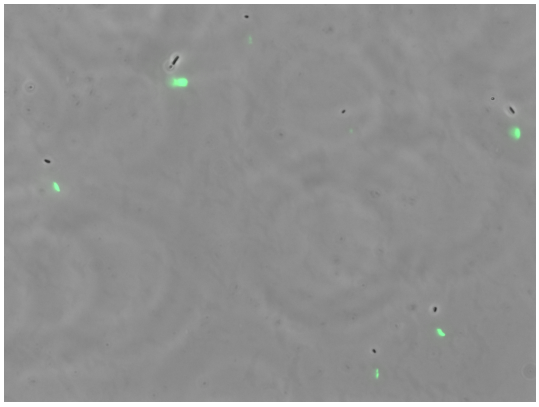


Snap-110.jpg

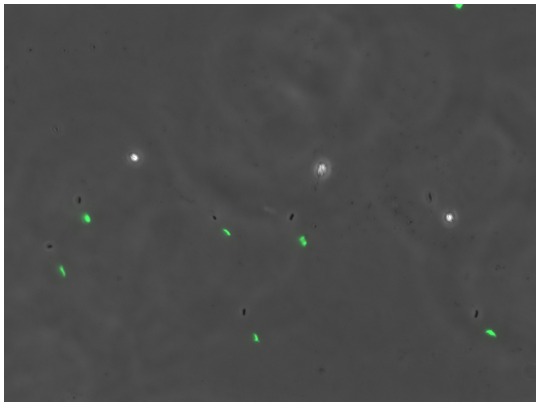


1:1000

Experiment-67.jpg



Experiment-66.jpg



10.9.2015

THURSDAY, 9/10

Petra

Resticting AtoB Gibson colony 6 to analyse on gel again:
AtoB Gibson 6 was the only AtoB-sample, that seems to possibly contain the right insert. To analyse it further and to get a better gel picture restricted the sample again with EcoRI and PstI.
For the restriction used 2,5 µl 10x Thermo Fisher Fast Digest Green Buffer and 1 µl Fast digest EcoRI & Fast digest PstI. The amounts of DNA and water pipetted are listed in Table 1. Total reaction volume: 25 µl. Incubated the reactions in 37 C for 20 min and inactivated in 80 C for 5 min.

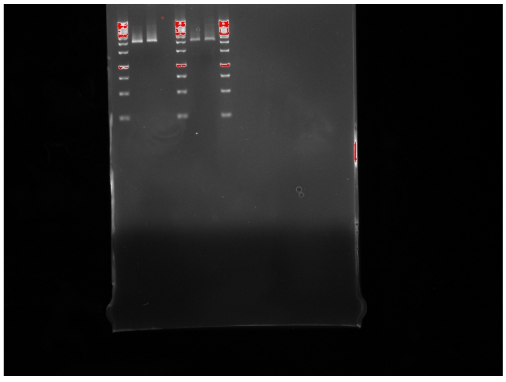
Table1

Construct	Concentration (ng/µl)	A260/A280	Amount pipetted for 400 ng (µl)	Water added (µl)
AtoB Gibson 6	36,7	1,89	10,8	9,7

Made a 1,2 % agarose gel with ETBR. Pipetted 6 µl and 12 µl samples of the AtoB restriction reaction made today to the wells. Used the name "new" for this restricted sample. Pipetted 6 µl and 12 µl samples of the AtoB restriction reaction made 8.9.15 to the wells. Used the name "old" for this restriction sample. Ran the old sample on this gel to compare the results. The old sample had been stored in RT for two days, which have to be taken into account if the results differ from the older gel picture, Used O'GeneRuler 1 kb ladder. Ran the gel for 1 h in 70 V. Pipetting order:

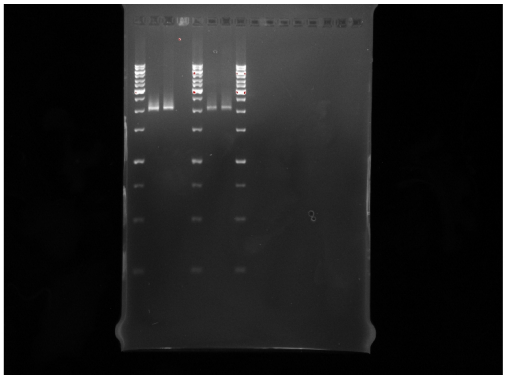
1. Ladder
2. AtoB Gibson 6 new, 6 µl
3. AtoB Gibson 6 new, 12 µl
4. -
5. Ladder
6. AtoB Gibson 6 old, 6 µl
7. AtoB Gibson 6 old, 12 µl
8. Ladder

Geldoc_2015-09-10_16hr_10min_AtoB_gibson_6_restriction_analysis_1h.jpg



The first gel picture was taken after 1h running. The desired 6400 bp insert can be seen above 2070 bp backcone. Continued the run for additional 1 h.

Geldoc_2015-09-10_17hr_44min_Atob_gibson_6_restriction_analysis_2h.jpg



The second gel pictur eafter running the gel for 2 h. The ladder has separated well but the 6400 bp insert can't be seen anymore. Decided to do a new colony PCR for AtoB Gibson 6 tomorrow.

There were not first colonies of new transformations of AH045+Amph and GFP+Amph. Because it was thought that the incubation time was too short for the temperature (RT), the plates were put for about altogether 6 hours in 37 C. In the end, there were white colonies in the plates, which also mean that buffer doesn't affect transformation. However, GFP+Amph transformation seems to have been failed because no green colonies couldn't be detected in UV. But to make sure, the GFP+Amph plates were put to incubate for o/n in RT.

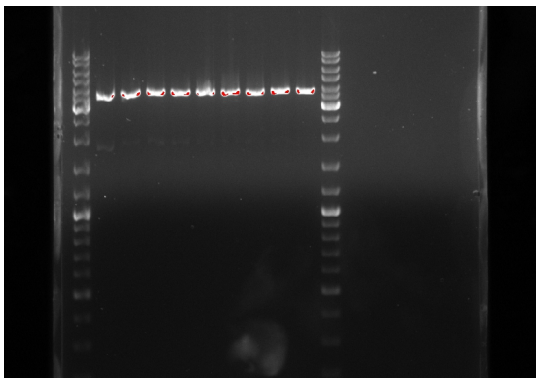
Because there were white colonies in the AH045+Amph plates, did o/n cultures of them in 2 ml LB with 2 ul AMP (chose total 9 different colonies -> 9 tubes).

Transformation of GFP+Amph and AH045+Amph, which were done on 8.9., were unsuccessful.

Restricted Car Amph (2nd gibson) with FD EcoRI & FD PstI. Followed mostly the protocol, but incubated 25 min and inactivated 10 min in 80 C.

Made a 1,3 % agarose gel with EtBr. Restricted Car Amph samples were run in the gel for 1 h with 100 V. Pipeting order was:
1. ladder 2 ul 2.-10. Car Amph 1-9 6 ul 11. ladder 2 ul

Geldoc_2015-09-10_17hr_57min_CarAmphgibson2.jpg



Results: None of the samples had Car Amph construct as it is over 5000 bp long.

11.9.2015

FRIDAY, 9/11

Petra

Analysing CAR in AH043, CAR in pSB1C3 and AtoB in pSB1C3 colonies with colony PCR:
Decided to analyse our propane constructs with colony PCR and use the primers of which one attach to the plasmid backbone and another to our desired insert.
Primers used:

- P001: forward primer, 20 bp, attach to the construct's prefix & following T7 promoter. Can be used for both CAR and AtoB. GC=55 %
- VR: Reverse primer for sequencing/amplifying BioBrick parts, 20 bp, Tm= 60 C according to parts.igem.org. GC=50 %

Made colony PCR for these colonies:
CAR:

- CAR gibson 2 (in AH043, an iGEM backbone with amp resistance)
- CAR gibson 3 (in AH043)
- CAR gibson 4 (in AH043)
- CAR gibson 7 (in AH043
- CAR gibson 2#1 (in pSB1C3, the biobrick backbone)
- CAR gibson 3#2 (in pSB1C3)
- CAR gibson 4#1 (in pSB1C3)

All the colonies above were the ones we sequenced.

In addition made colony-PCR also for these colonies, that looked promising in the gel picture mut weren't chosen to be sent to sequencing

- CAR gibson 2#2 (in pSB1C3)
- CAR gibson 3#1 (in pSB1C3)
- CAR gibson 4#2 (in pSB1C3)
- CAR gibson 7#1 (in pSB1C3)
- CAR gibson 7#2 (in pSB1C3)

AtoB

- AtoB gibson 6 (in pSB1C3)

In total made 13 colony Kapa PCR-reactions. Because there is so many sample colonies and our time in the lab is running out didn't do gradient PCR to test the right annealing temperature. Since annealing temperature is higher for P001 (higher GC) than for VR, decided to use Tm = 63 C for the primers.

Prepared all the DNA samples for the PCR reactions following Colony PCR protocol. Refreshed all the CAR colonies yesterday and used the refreshed cultures to make the samples. For AtoB gibson 6 sample used the sreening plate done on 2.9 when screening colonies with colony PCR.

14x KAPA reaction mix (322 µl)
213,5 µl water
70 µl 5x KAPAHiFi Fidelity Buffer
10,5 µl dNTP mix
10,5 µl P001 (10 mM)
10,5 µl VR (10 mM)
7 µl KAPAHiFi HotStart DNA Polymerase

Pipetted 23 µl reaction mix to 13 PCR tubes and added 2 µl sample DNA to each tube: total volume per tube = 25 µl

PCR protocol:
95 ºC - 4 min
98 ºC - 20 sec
63 ºC - 15 sec
72 ºC - 3 min
72 ºC - 4 min
Repeated underlined steps 25 times.

PCR results will be analysed on monday.

Miniprepped AH045+Amph following the kit protocol.
NanoDrop results:

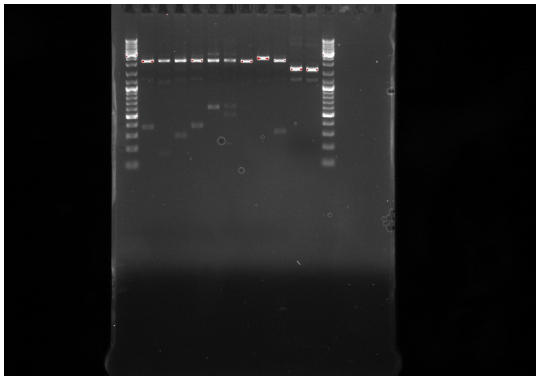
Table1

Sample	DNA (ng/ul)	A260/A280
Amph 1	29,8	1,88
Amph 2	33,3	1,88
Amph 3	29,1	1,94
Amph 4	22,6	1,85
Amph 5	31,7	1,88
Amph 6	50,1	1,96
Amph 7	35,3	1,91
Amph 8	40,7	1,87
Amph 9	31,6	1,84

Restricted AH045+Amph with FD EcoRI and FD PstI following the FastDigest restriction protocol for EcoRI incubating 25 min in 37 C and inactivating 10 min in 80 C.

Made a 1,3 % agarose gel with EtBr. Restricted AH045+Amph were run in the gel for 40 min with 100 V. Pipeting order was: 1.ladder 2 ul 2.-10. Amph 1-9 6 ul 11. Undigested Amph 1 6 ul 12. Undigested Amph 9 13. ladder 2 ul

Geldoc_2015-09-11_14hr_53min_AH045Amph.jpg



Results: There might be correct sized AH045+Amph insert in the 5th and 6th samples.
Did o/w cultures of GFP+Amph 1 & 2 and AH045+Amph 4, 5 & 6, which were made today, in 50 ml LB with 50 ul AMP. Also did o/w cultures of GFP+Amph 1 & 2 in 5 ml LB with 5 ul AMP. Incubated in a shaker in RT with 150 rpm.